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Resistance to Activated Protein C in Unselected Patients With Arterial and Venous Thrombosis

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Four hundred and ninety-three consecutive patients referred for arterial or venous thrombosis were screened for congenital and acquired abnormalities of blood coagulation predisposing to thrombosis, and were compared to 341 age- and sex-matched controls. The aim of the study was to determine the prevalence and clinical characteristics of resistance to activated protein C (APC), a defect shown to have different prevalences in different ethnic groups and to be associated with an increased risk of thrombosis. Seventy-three (15%) patients had both APC resistance and the 1691 G to A factor V gene mutation, compared to 6/341 (2%) controls. Seven patients had antithrombin deficiency (1.4%), 11 had protein C deficiency (2.2%), and 4 had protein S deficiency (0.8%). The relative risk of thrombosis in APC-resistant patients was 9.4. Resistance to APC was associated mainly with venous thrombosis, the most frequent being deep-vein thrombosis of the lower limbs. Fifty-eight percent of APC-resistant patients had an associated risk factor at the first thrombotic event: pregnancy and oral contraceptive intake were associated with the first thrombotic episode in 35% and 30% of women, respectively. APC resistance is the most frequent defect of blood coagulation in the general population and in the unselected thrombotic population studied by us. Am. J. Hematol. 55:59–64, 1997. © 1997 Wiley-Liss, Inc.

Key words: thrombosis; APC resistance; antithrombin; protein C; protein S

INTRODUCTION

APC resistance is common in individuals of European ancestry and is the most frequent inherited defect associated with idiopathic venous thromboembolism [1]. In the majority of cases, APC resistance is the phenotypic manifestation of a point mutation (G1691 to A) in the gene of coagulation factor V, which predicts an amino-acid substitution (Arg 506 to Gln) in one of the cleavage sites for APC [2]. The mutant factor V is degraded more slowly by APC, and this leads to less efficient inhibition of thrombin generation [3].

APC resistance can be diagnosed by functional assays that detect a shorter prolongation of clotting times induced by the addition of APC to plasma compared to normals [4]. In addition, the genetic defect can be unravelled by DNA techniques [2]. A very large variability in the prevalence of APC resistance has been observed both in patients with thrombosis (12–58%) [5–11] and in the general population (0–15%) [2,6,8–10,12–14). The sensitivity and specificity of the methods employed to diagnose APC resistance, the criteria used to select patient populations, and true ethnic variability all contribute to generate this wide range of prevalence values.

Few studies have analyzed in detail the clinical characteristics of thrombotic symptoms associated with APC resistance. The aim of our study was to analyze a large number of consecutive patients with arterial or venous thrombosis to determine the prevalence of APC resistance, the type of thrombotic symptoms associated with this defect, the risk factors associated with the first thrombotic event, and the relative risk of thrombosis of APC-resistant patients compared with a control group taken from the general population.

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MATERIALS AND METHODS

Patients and Controls

Five hundred and twenty-five consecutive patients with at least one objectively diagnosed episode of venous or arterial thrombosis were referred to the Thrombosis Center between January 1, 1993–September 30, 1995. Of these, a total of 32 (6%) patients were excluded from the study due to the presence of a prolonged APTT or a condition that did not allow a reliable estimate of APC resistance with the functional method used for screening. Of the 32 excluded patients, 3 were pregnant, 6 had liver, renal, or neoplastic disease, 6 had lupus anticoagulant, and 17 had other causes for a prolonged APTT. A total of 493 patients was included.

Deep-vein thrombosis was diagnosed by ultrasonography or venography; pulmonary embolism, by ventilation-perfusion scan or angiography; superficial thrombophlebitis, by ultrasonography or clinical observation; cerebral vein or arterial thrombosis, by angiography, computerized tomography (CT), or magnetic resonance imaging (MRI); visceral vein thrombosis, by ultrasonography or CT scan; and retinal vein thrombosis, by retinal fluorescein angiography. Acute myocardial infarction was diagnosed when an elevation of cardiac enzymes or specific alterations in the ECG or both were present; stroke was diagnosed by CT scan or MRI; and a transient ischemic attack was diagnosed when focal neurological signs appeared ex novo and disappeared within 24 hr, whether or not alterations of CT or MRI were detected.

The personal and family history of all patients was collected, and for each thrombotic event the diagnostic procedure used, the type and site of thrombosis, the presence of associated risk factors, and recurrences were documented. Pregnancy and the postpartum period, surgery, prolonged immobilization, trauma, oral contraceptive use, varicous veins (only for superficial thrombophlebitis), and intravenous drug therapy (only for superficial thrombophlebitis) were considered risk factors for venous thrombotic events. Hypertension (systolic blood pressure >160 and/or diastolic blood pressure >95 mm Hg, on at least two separate occasions), smoking (in excess of 10 cigarettes per day), dyslipidemia (>220 mg/dl blood cholesterol), overweight ($\geqslant$20% excess over ideal body weight), and hyperglycemia ($\geqslant$140 mg/dl fasting venous plasma glucose on at least two separate occasions) were considered risk factors for cardio- and cerebrovascular events.

Three hundred and forty-one individuals recruited from the same geographical community as the patients were studied as a control group. They had a negative personal history of thrombosis as assessed by a validated questionnaire [15], and were chosen as they matched the patients for sex and age.

In patients and controls the following laboratory tests were performed (in patients at least 3 months after the thrombotic event): prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, anticardiolipin antibodies, lupus anticoagulant, antithrombin, protein C, protein S, and APC resistance (see below for methodology). When an abnormal result was found, the test was repeated after 1 month for confirmation and a family study was performed. When an abnormal result of the functional assay for APC resistance was confirmed, DNA analysis to detect the factor V mutation was carried out.

Methods

APC resistance was measured using an APTT-based clotting assay slightly modified [7] from the original assay described by Dahlbäck et al. [4]. Specificity and sensitivity were 94% and 98%, respectively. Results were expressed as normalized ratios [7]. Search for the G1691-to-A mutation in the factor V gene was conducted by specific DNA amplification and digestion as described elsewhere [16]. Throughout this paper, the term ''APC-resistant'' refers to patients who have *both* APC resistance measured with the functional clotting test, *and* the factor V G1691-to-A mutation.

Antithrombin was measured by a commercially available functional assay (Coamatic Antithrombin, Chromogenix AB, Mölndal, Sweden) that evaluates its anti-Xa activity in the presence of heparin. In the presence of low values, anti-thrombin activity (in the presence and absence of heparin) as well as antigenic concentration were evaluated.

Protein C was measured by a functional clotting assay (IL Test ProClot, Instrumentation Laboratory S.p.A., Milan, Italy) after activation by snake venom (Protac®). In the presence of low values, amidolytic activity and antigenic concentration were also measured.

Concentration of total and free protein S were measured by ELISA with polyclonal anti-protein S antibodies [7]. Free protein S was separated from that bound to C4b-binding protein after precipitation of sample plasma with polyethylenglycol 6000 (3.5% final concentration).

Anticardiolipin antibodies were measured by ELISA as described elsewhere [17].

Statistical Analysis

Relative risk was determined as unadjusted odds ratios; 95% confidence intervals of prevalences and risk values were calculated by normal approximation to the Poisson distribution [18]. Statistical significance of the observed differences was determined by Fisher's test.

RESULTS

Characteristics of Consecutive Patients

Table I illustrates the types and prevalence of first thrombotic events that occurred in the 493 consecutive

TABLE I. Types of First Thrombotic Event in 493 Consecutive Patients With Venous and Arterial Thrombosis Referred to the Thrombosis Center Over a 33-Month Period

Type of event	No. of patients (%)
Deep-vein thrombosis	188 (38)
Stroke	77 (16)
Superficial thrombophlebitis	72 (5)
Transient ischemic attack	55 (11)
Myocardial infarction	29 (6)
Pulmonary embolism	24 (6)
Cerebral vein thrombosis	17 (4)
Retinal vein thrombosis	15 (4)
Visceral thrombosis[a]	11 (2)
Peripheral arterial thrombosis	4 (1)
Intracardiac thrombosis	1 (−)

[a]Two renal infarctions, 1 Budd-Chiari syndrome, 4 splenic and/or portal thromboses, 2 caval thromboses, 1 intestinal infarction, and 1 multiple placental infarction.

patients (303 women, 190 men, median age 39 years, range 1–75 years) included in this study. Median age at first event was 32 years (range, 0–73 years), 31 years in women (range, 0–66 years), and 37 years in men (range, 1–75 years). The most common event was deep-vein thrombosis of the lower limbs (38%), followed by ischemic stroke (16%). On the whole, 34% of referred patients had an arterial thrombotic event.

Associated risk factors at the first thrombotic episode were present in 301/493 (61%, 95% CI, 54–68%) of patients, and are shown in Table II. The two most common were oral contraceptive use (55% of women) and pregnancy and postpartum (19% of women).

Prevalence of APC Resistance and Clinical Characteristics of APC-Resistant Patients

Seventy-three (median age 42 years, range 19–71 years, 41 women, 32 men) of the 493 consecutive thrombotic patients had APC resistance according to our criteria (15%; 95% CI, 12–18%). Seventy-one were heterozygous for the mutation, and two were homozygous. Median age at the first thrombotic episode in APC-resistant patients was 29 years (range, 16–56 years). The types and sites of first thrombotic events are shown in Table III. The most frequent events were deep-vein thrombosis of the lower limbs and superficial thrombophlebitis. Arterial thrombotic events represented only 12% of the total. Fifty-eight percent of patients (42/73) had an associated risk factor at the first thrombotic event. The most frequent factors (Table IV) were pregnancy and postpartum (35% of women), followed by oral contraceptive use (32% of women) and trauma (13% of women).

Prevalence of Other Defects and Associations With APC Resistance

Seven of the 493 (1.4%, 95% CI, 0.6–3%), 11/493 (2.2%, 95% CI, 1–4%), and 4/493 (0.8%, 95% CI, 0.2–2%) consecutive patients had a deficiency of antithrombin, protein C, and protein S respectively. None of the 493 patients were positive for anticardiolipin antibodies (defined as the presence of anti-cardiolipin IgG >10 units, confirmed once after at least 8 weeks) [17].

One of the antithrombin-deficient patients was also

TABLE II. Risk Factors Associated With First Thrombotic Event in 493 Consecutive Patients With Venous and Arterial Thrombosis Referred to the Thrombosis Center Over a 33-Month Period

Risk factor	No. of patients (%)
Oral contraceptives	111 (55)[a]
Pregnancy and postpartum	39 (19)[a]
Smoking	38 (13)
Surgery	33 (11)
Trauma and immobilization	31 (10)
Dyslipidemia + smoking + hypertension	20 (7)
Varicose veins	10 (3)
Intravenous drug therapy	7 (2)
Hypertension	7 (2)
Diabetes[b]	3 (1)
Dyslipidemia	1 (<1)
Overweight	1 (<1)

[a]Percent is calculated on the total number of women having an associated risk factor.
[b]Two type I and 1 type II diabetes mellitus.

TABLE III. Types of First Thrombotic Event in 73 APC-Resistant Patients

Type of event	No. of patients (%)
Deep-vein thrombosis of the lower limbs	42 (58)
Superficial thrombophlebitis	17 (23)
Stroke	4 (5)
Cerebral vein thrombosis	3 (4)
Transient ischemic attacks	3 (4)
Myocardial infarction	2 (3)
Pulmonary embolism	1 (1)
Visceral vein thrombosis	1 (1)

TABLE IV. Risk Factors Associated With First Thrombotic Event in 73 APC-Resistant Patients

Risk factor	No. of patients (%)
Pregnancy and postpartum	13 (35)[a]
Oral contraceptives	12 (32)[a]
Trauma	6 (13)
Varicose veins	4 (9)
Surgery	3 (7)
Smoking	3 (7)
Prolonged immobilization	2 (4)
Hypertension	1 (2)
Intravenous drug therapy	1 (2)

[a]Percent is calculated on the total number of women having an associated risk factor.

APC-resistant. For this patient (a 30-year-old woman), a family study was not feasible. Her thrombotic symptoms were severe, i.e., cerebral vein thrombosis at age 29 while she was on oral contraceptives for 3 months and, at the same time, deep-vein thrombosis of a lower limb, complicated by pulmonary embolism.

Relative Risk of Thrombosis in APC-Resistant Patients

To determine the relative risk of thrombosis in APC-resistant patients compared to nonresistant individuals, 341 sex- and age-matched controls were chosen from the general population. Six of 341 controls were resistant to APC and were heterozygous for the G1691-to-A mutation with a prevalence of 2% (95% CI, 1–4%). The relative risk of thrombosis for heterozygotes, calculated as unadjusted odds ratio, was 9.4 (95% CI, 4.1–22.0%, $P < 0.0001$).

Prevalence of APC Resistance in Patients with Early-Onset Venous Thrombosis

The 493 patients included in our study were not selected for age of onset nor for type of first thrombotic event. Since these features of the cohort may have contributed to the relatively low prevalence of APC resistance found, we selected a subgroup of consecutive patients who had early-onset venous thromboembolism, i.e., a first *venous* thrombotic event *before age 45*. There were 256 patients fulfilling these criteria (178 women, 78 men, median age 35 years, range 1–71 years). The main thrombotic events in these patients were deep-vein thrombosis of the lower limbs (61%) and superficial thrombophlebitis (21%). Median age at onset of first thrombotic episode was 28 years (range, 1–44 years). Prevalence of APC resistance in this selected subgroup was 20% (51/256, 95% CI, 15–26%).

DISCUSSION

We chose to analyze a large unselected population with arterial and venous thrombosis to determine the prevalence of APC resistance and to characterize, without preselection, the type of thrombotic episodes associated with APC resistance. A relatively small number of consecutive patients was excluded from the study due to the presence of a prolonged APTT or a condition (such as pregnancy or lupus anticoagulant) that did not allow a reliable estimate of APC resistance with the functional assay used for screening.

The Thrombosis Center of our hospital is a tertiary referral center which receives patients mainly from Northern Italy, though referrals from Central and Southern Italy are not uncommon. Patients are generally referred by their family physician or from district hospitals. In our cohort, the median age at first event was relatively young, but the wide range indicates that older patients are also referred. There were more women than men, and this may in part explain why the most relevant risk factors associated with the first event were oral contraceptive use, pregnancy, and postpartum. As anticipated, the most frequent defect found in our series was APC resistance. Among 73 patients with this defect, the most frequent thrombotic events were deep-vein thrombosis of the lower limbs and superficial thrombophlebitis. Cerebral vein thrombosis, which has recently been shown to be associated with APC resistance [19], was found in 4% of patients. Onset of thrombosis occurred at a young age, the range being narrower (range, 16–56 years) than for the 493 consecutive patients (range, 0–73 years).

Only 12% of total events in APC-resistant patients were arterial, compared to the 34% of the consecutive patients. It is still debated whether resistance to APC is associated with arterial thrombosis. The majority of clinical studies [1,9,10,14], one of which was prospective [10], indicate that APC resistance is not associated with myocardial infarction or cerebrovascular disease, though a recent report shows a relatively high prevalence of the factor V mutation in young women who smoke and have myocardial infarction [20]. In our center, a retrospective study of patients with juvenile stroke suggests that only cryptogenetic stroke may be associated with a higher prevalence of the factor V mutation [21]. The association of APC resistance with peripheral arterial occlusion has not been extensively evaluated.

Age of onset and distribution of symptoms in APC-resistant patients were very similar to those observed in the congenital defects of protein C and protein S [22]. Independent risk factors most frequently associated with the first thrombotic episode were pregnancy, postpartum, and oral contraceptive use. Pregnancy and postpartum are also associated with a high prevalence of thrombosis in women deficient in antithrombin, protein C, and protein S [23,24]. More than half of APC-resistant women appear to experience their first thrombotic event during pregnancy [25–27]. Hence, the usefulness of screening for the factor V mutation in pregnant women should perhaps be evaluated. The association of oral contraceptive use with APC resistance confers a relative risk for thrombosis of approximately 34 compared to nonresistant women who do not use oral contraceptives [28]. This impressive figure and the frequent finding of oral contraceptive use as an independent risk factor associated with the first thrombotic episode in APC resistance [1,9,12,28], confirmed also in our series, pose the question of the usefulness of screening for APC resistance before starting oral contraceptives. A recent report suggests that screening for APC resistance before starting oral contraceptives might not be cost-effective [29]. This report deals only with fatal thrombotic events. An evaluation of cost-effectiveness should be also carried out con-

sidering nonfatal thrombotic events, which are more frequent, and taking into account the peculiar epidemiological, social, and public-health aspects of each country.

The 15% prevalence of APC resistance found in this study is at the lower end of the range of values reported in the literature (12–58%). This is likely to be due both to the relatively low prevalence of APC resistance in Italy (2% in our control group) and to the fact that our cohort was not selected for age or type of thrombotic event. On the other hand, when patients were selected for type of thrombosis (only venous) and for age of onset (<45 years old), the prevalence of APC resistance was 20%. This confirms that APC resistance is a risk factor for venous thrombosis in the young. It has been shown that APC resistance is a risk factor also for older patients [10,30]. Accordingly, we found 22/237 (9%) APC-resistant patients with a first event after age 45 years.

From our study, APC-resistant patients have a relative risk of developing thrombosis which is approximately nine times that of nonresistant individuals, a figure consistent with those reported by other groups for heterozygotes [2,6,9]. Homozygotes were excluded from this calculation, but due to their low number, no separate evaluation of risk was possible.

We found only one resistant patient with an associated deficiency of a naturally occurring anticoagulant, i.e., antithrombin. Because a family study was not possible, we could not study segregation patterns and development of symptoms in patients with dual deficiencies. However, this patient had a very severe thrombotic history, in agreement with what has been reported in the literature for patients with dual deficiencies [31–33].

CONCLUSIONS

APC resistance associated with the factor V G1691-to-A mutation is frequent in the general population and in unselected patients with thrombosis in Italy. APC resistance conveys a ninefold higher risk of developing thrombosis than that of individuals without the mutation. Symptoms are similar to those observed in hereditary thrombophilic syndromes due to deficiencies of the protein C pathway. Since the most frequent risk factors associated with the first thrombotic event are oral contraceptive use and pregnancy and postpartum, evaluation of cost-effectiveness of screening for APC resistance in women is suggested.

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